



Characterisation of water-extractable soil organic phosphorus by phosphatase hydrolysis

Benjamin L. Turner^{a,b,*}, Ian D. McKelvie^a, Philip M. Haygarth^b

^aWater Studies Centre and Chemistry Department, Monash University, Clayton 3800, Victoria, Australia

^bInstitute of Grassland and Environmental Research, North Wyke, Okehampton, Devon EX20 2SB, UK

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Abstract

Information on the chemical forms of organic phosphorus (P) in soil waters is fundamental to understanding the dynamics of soil organic P and its potential for transfer from soils to watercourses. Phosphatase enzymes were used to classify water-extractable molybdate-unreactive P (MUP) from five Australian pasture soils into compounds that could be hydrolysed by (i) alkaline phosphomonoesterase (comprising labile orthophosphate monoesters, such as sugar phosphates), (ii) a combination of phosphodiesterase and alkaline phosphomonoesterase (comprising labile orthophosphate monoesters and orthophosphate diesters, such as nucleic acids and phospholipids), and (iii) phytase (including inositol hexakisphosphate). The phosphomonoesterase and phosphodiesterase preparations were specific to the target substrates, but the phytase preparation hydrolysed all ester-P bonds. Air drying of soils increased the amounts of water-extractable MUP from between 0.15 and 0.45 $\mu\text{g P g}^{-1}$ in extracts of moist soils to between 1.04 and 1.63 $\mu\text{g P g}^{-1}$ in extracts of dry soils. Only small amounts of the MUP were hydrolysed by phosphomonoesterase alone (mean 5.6%), whilst a combination of phosphomonoesterase and phosphodiesterase hydrolysed much greater proportions (6–63%). This suggested the dominance of orthophosphate diesters in grassland soil solutions. The phytase preparation hydrolysed large proportions of MUP in extracts of dry soils (33–49%), suggesting the release of enzyme-hydrolysable inositol hexakisphosphate to water following the rapid rewetting of dry soils. The large proportions of MUP that remained unhydrolysed in all extracts probably consisted of microbial cell debris and high molecular weight P-containing compounds. The phosphatase technique is a simple and accurate method for determining functional classes of MUP in soil waters. Published by Elsevier Science Ltd.

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1. Introduction

The transfer of phosphorus (P) in drainage from agricultural soils to watercourses can contribute to toxic algal blooms and other water quality problems associated with eutrophication (Foy and Withers, 1995). Organic P is an important component of P transfer, because it can constitute a large proportion of the total P in soil solution (Shand and Smith, 1997), leachate (Turner and Haygarth, 2000) and overland flow (Haygarth and Jarvis, 1997), and contributes to algal growth through the release of orthophosphate by phosphatase enzymes (Whitton et al., 1991). Despite the importance of organic P in the P transfer process, it remains poorly understood (Frossard et al., 2000). Total P in soil

waters is classified operationally by reaction with molybdate; molybdate-reactive P (MRP) approximates inorganic orthophosphate (although some acid-hydrolysis of condensed and organic P compounds can occur), whilst molybdate-unreactive P (MUP) includes both organic and condensed P compounds (Shand and Smith, 1997). The MUP fraction contains a multitude of chemical forms that behave differently in the soil environment, especially in terms of their availability to plants, resistance to degradation by soil enzymes (Bowman and Cole, 1978), mobility in the soil (Frossard et al., 1989) and availability to algae in watercourses (Whitton et al., 1991). Understanding these processes requires information on the chemical nature of organic P compounds. However, the low concentrations present in soil waters precludes most techniques for their separation and detection and only a few compounds have been positively identified (Wild and Oke, 1966; Espinosa et al., 1999). Espinosa et al. (1999) developed a method using strong anion exchange resins and high-performance liquid chromatography to pre-concentrate and separate trace

* Corresponding author at United States Department of Agriculture, Agricultural Research Service, Northwest Irrigation and Soils Research Laboratory, 3793N-3600E, Kimberly, ID 83341, USA. Tel.: +1-208-423-6524; fax: +1-208-423-6555.

E-mail address: bturner@nwisrl.ars.usda.gov (B.L. Turner).

Table 1
Phosphatase enzymes and buffers used to determine functional classes of molybdate-unreactive P in soil water-extracts

Enzyme	Type	Source	Specified activity	Sigma No.	Buffer	Activity of preparation
Alkaline phosphatase (EC 3.1.3.2.)	Type III chromatographically purified	<i>Escherichia coli</i>	60 units mg ⁻¹ protein (3.6 mg protein ml ⁻¹)	P-4252	0.1 M Tris-HCl pH 8.0	1 unit ml ⁻¹
Phosphodiesterase ^a (EC 3.1.4.1.)	Phosphodiesterase 1, Type IV	<i>Crotalus atrox</i> venom	0.03 units mg ⁻¹ solid	P-4506	0.1 M Tris-HCl pH 8.8	0.03 units ml ⁻¹
Phytase (EC 3.1.3.8.)	myo-inositol hexakisphosphate 3-phosphohydrolase	<i>Aspergillus ficuum</i>	3.5 units mg ⁻¹ solid	P-9792	0.1 M Glycine-HCl pH 2.5	1 unit ml ⁻¹

^a The preparation used in the phosphatase hydrolysable P assays included alkaline phosphomonoesterase (see Section 2.2).

organic P compounds in soil leachate, identifying inositol hexakisphosphate, sugar and condensed phosphates and phosphonate. However, such techniques are still in the developmental stages and cannot readily analyse large number of environmental samples at low cost.

An alternative technique uses phosphatase enzymes to characterise functional classes of organic P compounds in soil waters. This technique has been widely applied in studies of aquatic organic P cycling (e.g. Hino, 1989; Feuillade and Dorioz, 1992) and has recently been applied to soil waters (Fox and Comerford, 1992; Pant et al., 1994; Shand and Smith, 1997; Hayes et al., 2000). However, the technique suffers from poor substrate specificity of some commercial phosphatase preparations, which prevents the identification of even broad classes of organic P compounds (Shand and Smith, 1997). Furthermore, no attempts have been made to use phosphodiesterase, despite the importance of orthophosphate diesters in the soil P cycle (Stewart and Tiessen, 1987).

Phosphorus that is potentially transferable from soil to water can be conveniently estimated by soil water-extraction (Chapman et al., 1997; McDowell and Sharpley, 2001; Turner and Haygarth, 2001), which allows P solubilisation to be investigated separately from the complex hydrological factors that control soluble P concentrations and forms under field conditions. These tests often focus on MRP (e.g. McDowell and Sharpley, 2001) and little is known about water-soluble MUP compounds, despite the fact that they can represent >90% of the total extracted P (Turner and Haygarth, 2001). The aims of this work were to address the methodological issues of the phosphatase hydrolysis technique (substrate specificity and the use of phosphodiesterase) and to identify functional classes of potentially mobile organic P in soil water-extracts.

2. Materials and methods

2.1. Principle of the phosphatase hydrolysis technique

The addition of commercially available phosphatase enzymes to an aqueous sample releases orthophosphate from organic and condensed P compounds by hydrolysis, which can be determined by standard analytical procedures. The use of substrate-specific phosphatase enzymes allows the hydrolysable MUP to be classified into several functional groups.

2.2. Enzymes and buffers

Alkaline phosphomonoesterase, phosphodiesterase and phytase (Sigma Chemicals) were dissolved in the appropriate buffers (Table 1). All buffers contained 2 mM magnesium chloride (MgCl₂) (Feuillade and Dorioz, 1992), because magnesium ions (Mg²⁺) are natural activators of most enzymes acting on phosphorylated compounds (Dixon and Webb, 1966). Alkaline phosphomonoesterase was used

Table 2

Physico-chemical properties of the five Australian grassland soils used to study functional classes of molybdate-unreactive P in soil water-extracts

	Soil reference number				
	1	2	3	4	5
USDA soil type ^a	Haplustult	Haplustalf	Haplustalf	Haplustox	Haplustox
Topsoil texture ^b	sandy silt loam	silty clay loam	clay loam	clay	clay
pH (water)	5.0	5.0	5.1	5.1	4.8
Mineral P fertilizer (kg ha ⁻¹ y ⁻¹)	35	43	28	140	0
<i>Textural information (%)</i>					
Sand (2000–63 µm)	33	13	41	11	8
Silt (63–2 µm)	49	58	33	32	26
Clay (<2 µm)	18	29	26	57	66
<i>Total soil nutrients (mg g⁻¹ dry soil)</i>					
Carbon	62.1	58.4	45.7	97.4	103
Nitrogen	5.42	5.69	4.91	8.87	9.90
Phosphorus	0.78	1.16	0.68	2.10	1.80
<i>NaHCO₃-extractable P (µg g⁻¹ dry soil)</i>					
Inorganic	27	27	4.7	13	3.2
Organic	17	16	15	18	18

^a United States Department of Agriculture classification based on the whole soil profile.^b Based on soil sampled from the top 7.5 cm.

in the phosphodiesterase preparation, because studies on aquatic environments have shown that phosphodiesterase alone hydrolyses only one ester-P bond on the diester molecule. This leaves an orthophosphate monoester, which requires the presence of phosphomonoesterase to complete the release of orthophosphate (Hino, 1989; Feuillade and Dorioz, 1992). The presence of enzyme protein can cause a precipitate to form during P detection (Shand and Smith, 1997), so enzyme concentrations were kept as low as possible. The phytase preparation was centrifuged for 10 min at approximately 1500 *g* to remove particulate material. The presence of enzymes at the concentrations used here did not interfere with MRP detection, confirmed by running orthophosphate standards containing the enzymes.

2.3. Assay procedure

The assay mixture consisted of 4.5 ml model compound or soil water-extract and 0.25 ml of 0.1 M sodium azide (NaN₃, 5 mM final concentration) to prevent microbial interference during the assay (Feuillade and Dorioz, 1992). The assay was initiated by the addition of 0.25 ml enzyme mixture (either alkaline phosphomonoesterase, alkaline phosphomonoesterase + phosphodiesterase or phytase) in the appropriate buffer (final buffer concentration in the assay mix was 5 mM buffer and 100 µM MgCl₂). The mixtures were incubated for 16 h at 37°C in 25 ml plastic centrifuge tubes. Previous studies showed that assay times between 6 and 12 h achieved maximum MUP hydrolysis in soil waters (Pant et al., 1994; Shand and Smith, 1997; Hayes et al., 2000). The MUP hydrolysed by each enzyme preparation was calculated by subtracting the MRP prior to incubation from the MRP after incubation. Results were corrected

for blanks, which received enzyme-free buffer, and for MRP in the enzyme preparations.

2.4. Substrate specificity of the phosphatase preparations

The substrate specificity of the enzyme preparations was investigated for a range of P compounds, including orthophosphate monoesters (inositol hexakisphosphate, glucose-6-phosphate, *para*-nitrophenyl phosphate), orthophosphate diesters (DNA, bis-*para*-nitrophenyl phosphate), condensed-P compounds (*tetra*-sodium pyrophosphate, adenosine 5'-triphosphate) and phosphonate (±2-aminoethyl phosphonic acid) purchased from Sigma Chemicals. The model compounds (1 mg P l⁻¹) were incubated in triplicate for 16 h at 37°C with buffered enzyme mixtures and analysed for MRP. Controls contained enzyme-free buffer and represented chemical (non-enzymatic) degradation, which can include UV oxidation or mineral-mediated hydrolysis (Francko and Heath, 1979; Baldwin et al., 1995). Acid-induced hydrolysis of P compounds during P detection by the molybdate reaction was accounted for by determining MRP in the model compound solutions prior to incubation and subtracting this from the final MRP concentration.

2.5. Soil collection, description and water extraction

Five pasture soils containing a range of clay (18–66%), total carbon (46–103 mg g⁻¹ dry soil) and total P concentrations (0.68–2.10 mg g⁻¹ dry soil) were sampled to a depth of 7.5 cm from experimental sites near the Agriculture Victoria Research Institute, Ellinbank, Victoria, Australia (Table 2). The sites are used for monitoring overland flow as part of an ongoing research project and consist of 1–2 ha pasture paddocks (Nash and Murdoch, 1997). The soils were sieved <2 mm and stored at 4°C until use.

Table 3

Recovery of P (%) from model P compounds (1 mg l^{-1}) by enzymatic and chemical hydrolysis. Vales are means of triplicate samples

Compound	pH 2.5 buffer	pH 8.0 buffer	Alkaline phosphomonoesterase	Alkaline phosphomonoesterase + phosphodiesterase	Phytase
Compound recovery as MRP (%)					
\pm 2-aminoethylphosphonic acid	< 0.1	0.1	2.9	0.9	0.7
Adenosine 5'-triphosphate	< 0.1	< 0.1	97.3	^a	98.4
bis-(<i>para</i> -nitrophenyl) phosphate	< 0.1	< 0.1	2.4	19.3	80.7
Deoxyribonucleic acid	< 0.1	< 0.1	3.2	88.4	90.3
Glucose-6-phosphate	< 0.1	< 0.1	99.1	^a	95.2
Inositol hexakisphosphate	0.8	0.9	5.4	3.4	95.9
<i>para</i> -nitrophenyl phosphate	2.4	0.6	98.9	^a	96.8
<i>tetra</i> -sodium pyrophosphate	0.6	< 0.1	98.6	^a	95.0

^a See the result for alkaline phosphomonoesterase.

Soils were prepared in the moist and dry states, because dry soils were suspected to release more and different organic P compounds to water than moist soils (Turner and Haygarth, 2001). Soils were dry when sampled, so were re-moistened to approximately 33% gravimetric water content and allowed to equilibrate in the dark at 20°C for 10 days (Brookes et al., 1984). These were designated as 'moist' soils. Subsamples of these soils were air-dried for 7 days at 30°C on shallow metal trays and designated 'dry' soils.

Moist and dry soils (10 g dry soil basis) were extracted in triplicate with deionised water at a 4:1 solution-to-soil ratio in 50 ml plastic centrifuge tubes by shaking end-over-end for 1 h. The unit amount of extracted organic P remains constant as the extract ratio widens (Chapman et al., 1997), so the extraction ratio was chosen to give concentrations of organic P in solution at which changes due to enzymatic hydrolysis would be detectable (between 50 and $500 \mu\text{g l}^{-1}$). Water-extracts were centrifuged for 15 min at approximately 10,000 *g* and filtered through $0.45 \mu\text{m}$ membranes (Activon MFG 110517 syringe filters). Triplicate extracts of each soil were carried through individually as replicates for the phosphatase hydrolysable P assays.

2.6. Analytical

Molybdate-reactive P was determined by flow injection analysis using an in-house system with P detection at 690 nm (Karlberg and Pacey, 1989). Total P in the samples was determined after sulphuric acid-nitric acid digestion with P detection at 880 nm (APHA-AWWA-WPCF, 1998). Molybdate-unreactive P was calculated by the difference between total P and MRP. The rapid nature of the flow injection analysis procedure used here (contact time <1 min), minimises the acid-induced breakdown of organic and condensed P compounds that can occur during batch analysis procedures (Dick and Tabatabai, 1977).

Soils were analysed for total C and N using a Carlo Erba

model NA2000 elemental analyser. Total soil P was determined by sodium hydroxide fusion (Smith and Bain, 1982), and pH was measured in a 1:2.5 soil-to-deionised water suspension using a glass electrode. NaHCO_3 extractable P was determined by the method of Olsen et al. (1954), with organic P determined by difference between total P (determined following digestion as above) and inorganic P.

3. Results

3.1. Substrate specificity

Alkaline phosphomonoesterase was specific to the target substrates, giving approximately 100% recovery of condensed P compounds and labile orthophosphate monoesters (adenosine 5'-triphosphate, glucose-6-phosphate, *para*-nitrophenyl phosphate and *tetra* sodium pyrophosphate), but showing negligible activity towards \pm 2-aminoethyl phosphonic acid, inositol hexakisphosphate and orthophosphate diesters (Table 3). The small amounts of activity detected towards inositol hexakisphosphate (5.4%) could indicate the presence of lower inositol phosphates in the model compound preparation, which would be amenable to hydrolysis by phosphomonoesterase.

The alkaline phosphomonoesterase + phosphodiesterase combination released orthophosphate from orthophosphate diesters, but did not give 100% recoveries. Only 19% of bis-*para*-nitrophenyl phosphate was hydrolysed, although 88% of the orthophosphate was released from DNA. No activity was detected towards \pm 2-aminoethyl phosphonic acid or inositol hexakisphosphate.

The phytase preparation gave approximately 100% recovery of orthophosphate from inositol hexakisphosphate, but also hydrolysed condensed phosphates, labile orthophosphate monoesters and orthophosphate diesters. The preparation hydrolysed DNA to a similar extent to the alkaline phosphomonoesterase + phosphodiesterase preparation, but hydrolysed a greater proportion of the bis-*para*-nitrophenyl

Table 4
Amounts of P fractions extracted by water from moist and air-dried soils. See Table 2 for soil information. Values are means \pm standard error of triplicate extracts. Values in parentheses are the proportion of total P as molybdate-unreactive P

Soil no.	Total P				Reactive P				Unreactive P			
	Moist ($\mu\text{g g}^{-1}$ dry soil)	Dry ($\mu\text{g g}^{-1}$ dry soil)	Difference (%)	Moist ($\mu\text{g g}^{-1}$ dry soil)	Dry ($\mu\text{g g}^{-1}$ dry soil)	Difference (%)	Moist ($\mu\text{g g}^{-1}$ dry soil)	Dry ($\mu\text{g g}^{-1}$ dry soil)	Moist ($\mu\text{g g}^{-1}$ dry soil)	Dry ($\mu\text{g g}^{-1}$ dry soil)	Difference (%)	Difference (%)
1	2.79 \pm 0.02	4.37 \pm 0.15	56	2.34 \pm 0.00	2.87 \pm 0.01	22	0.45 \pm 0.03 (16)	1.50 \pm 0.15 (34)	0.45 \pm 0.03 (16)	1.50 \pm 0.15 (34)	232	232
2	2.81 \pm 0.04	4.57 \pm 0.04	63	2.51 \pm 0.03	3.09 \pm 0.07	23	0.34 \pm 0.03 (12)	1.47 \pm 0.08 (32)	0.34 \pm 0.03 (12)	1.47 \pm 0.08 (32)	337	337
3	0.29 \pm 0.03	1.88 \pm 0.05	545	0.11 \pm 0.00	0.25 \pm 0.01	132	0.15 \pm 0.01 (51)	1.63 \pm 0.05 (87)	0.15 \pm 0.01 (51)	1.63 \pm 0.05 (87)	995	995
4	0.95 \pm 0.04	1.37 \pm 0.04	45	0.53 \pm 0.01	0.33 \pm 0.01	–37	0.42 \pm 0.05 (44)	1.04 \pm 0.04 (76)	0.42 \pm 0.05 (44)	1.04 \pm 0.04 (76)	149	149
5	0.48 \pm 0.02	1.38 \pm 0.11	187	0.24 \pm 0.01	0.14 \pm 0.01	–44	0.24 \pm 0.01 (49)	1.24 \pm 0.11 (90)	0.24 \pm 0.01 (49)	1.24 \pm 0.11 (90)	425	425

phosphate (81%). No hydrolysis of \pm 2-aminoethyl phosphonic acid was detected by the phytase preparation.

Negligible chemical hydrolysis (<1%) was detected in controls containing buffer alone at pH 2.5 or 8.8 except for *para*-nitrophenyl phosphate, which degraded slightly (2.4%) in the acidic buffer (Table 3).

3.2. Phosphorus composition of soil water extracts

More P was extracted in water from dry soils than from moist soils (Table 4). Water-extractable total P ranged between 0.29 and 2.81 $\mu\text{g P g}^{-1}$ from moist soil to between 1.37 and 4.57 $\mu\text{g P g}^{-1}$ from dry soil, equivalent to increases after drying of between 45 and 545%. Similarly, greater amounts of MUP were extracted from all soils after drying, ranging between 0.15 and 0.45 $\mu\text{g P g}^{-1}$ from moist soils, to between 1.04 and 1.63 $\mu\text{g P g}^{-1}$ from dry soils, equivalent to increases of between 149 and 995%. Water-extractable MRP ranged between 0.11 and 2.51 $\mu\text{g P g}^{-1}$ from moist soils and between 0.14 and 3.09 $\mu\text{g P g}^{-1}$ from dry soils, but did not increase after drying in two clay soils with low water-extractable MRP. The proportion of the total extracted P as MUP increased following soil drying, from between 12 and 51% in moist soils, to between 32 and 90% in dry soils. Concentrations of MUP ranged between 37 and 113 $\mu\text{g P l}^{-1}$ in extracts of moist soils to between 260 and 407 $\mu\text{g P l}^{-1}$ in extracts of dry soils, indicating that there was no requirement for pre-concentration prior to the phosphatase technique.

3.3. Phosphatase hydrolysable phosphorus in soil water extracts

Greater amounts of MUP were hydrolysed in water-extracts of dry soils compared to moist soils, which was expected due to the larger amounts of MUP extracted from dry soils. However, there did not appear to be any clear trends in the amounts of hydrolysable P fractions amongst the different soils (Fig. 1). Alkaline phosphomonoesterase alone hydrolysed only small amounts of MUP from water-extracts of all soils, although greater proportions were hydrolysed in moist soils (0–20%) compared to dry soils (<5%). By comparison, the alkaline phosphomonoesterase + phosphodiesterase combination hydrolysed greater proportions of MUP in extracts of moist soils (6–63%) and dry soils (9–28%), although the latter represented greater absolute amounts. In some extracts of moist soils, the phytase preparation hydrolysed less MUP than the other preparations (soils 1, 4 and 5), despite hydrolysing greater proportions of model compounds. However, the phytase preparation hydrolysed large and consistent proportions of MUP in extracts of dry soils (33–49%).

By assuming, on the basis of the substrate specificity tests, that the alkaline phosphomonoesterase + phosphodiesterase combination hydrolysed the same compounds as alkaline phosphomonoesterase alone, and similarly that the phytase preparation hydrolysed the same compounds as

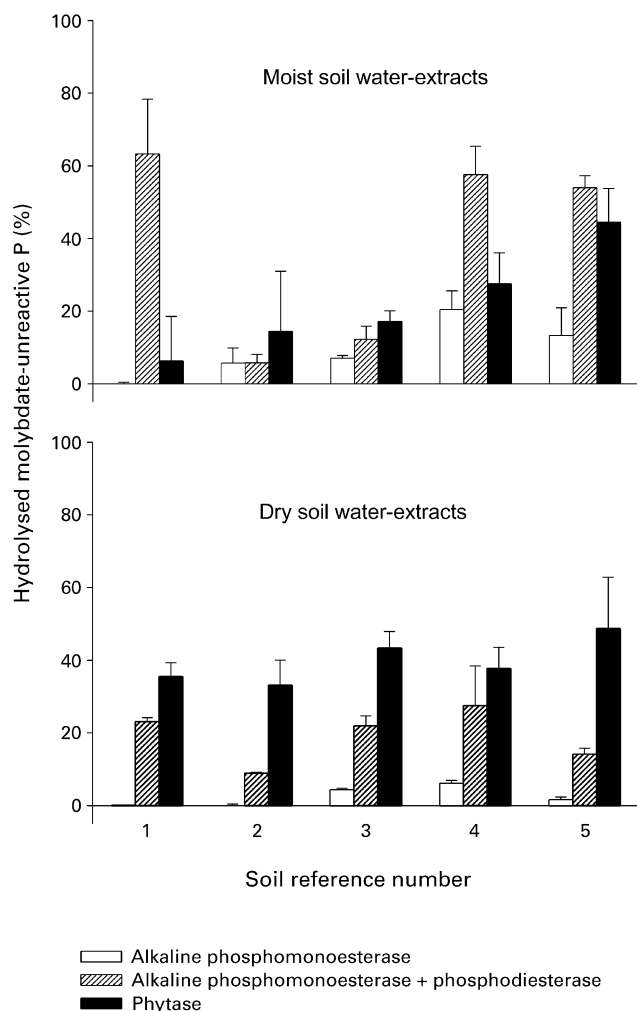


Fig. 1. Hydrolysis of molybdate-unreactive P (%) by phosphatase enzymes in water extracts of moist and air-dried soils. Values are means \pm standard error of triplicate extracts.

alkaline phosphomonoesterase and the alkaline phosphomonoesterase + phosphodiesterase combination in addition to inositol hexakisphosphate, it was possible to classify the hydrolysed MUP into functional groups (Table 5). These were

1. labile orthophosphate monoesters (hydrolysed by alkaline phosphomonoesterase);

2. orthophosphate diesters (alkaline phosphomonoesterase + phosphodiesterase-hydrolysed P minus labile orthophosphate monoesters);
3. inositol hexakisphosphate (phytase-hydrolysed P minus orthophosphate diesters and labile orthophosphate monoesters).

On this basis, the amounts of functionally classified water-extractable P compounds in dry soils were: labile orthophosphate monoesters $0\text{--}0.072 \mu\text{g P g}^{-1}$, orthophosphate diesters $0.132\text{--}0.344 \mu\text{g P g}^{-1}$, inositol hexakisphosphate $0.112\text{--}0.416 \mu\text{g P g}^{-1}$. The inositol hexakisphosphate could only be calculated for extracts of dry soils, because of the apparent inhibition of phytase in some extracts of moist soils. However, the equivalent moist-soil concentrations for labile orthophosphate monoesters were $0\text{--}0.08 \mu\text{g P g}^{-1}$ and for orthophosphate diesters were $0\text{--}0.276 \mu\text{g P g}^{-1}$.

4. Discussion

4.1. Substrate specificity of the phosphatase preparations

The alkaline phosphomonoesterase and phosphodiesterase preparations were specific to the target compounds, hydrolysing labile monoester and diester P bonds, respectively. The poor recovery of bis-*para*-nitrophenyl phosphate by the alkaline phosphomonoesterase + phosphodiesterase combination may be an artefact of the synthetic nature of this compound, because hydrolytic activity was satisfactory towards the natural substrate DNA. This raises questions about the use of bis-*para*-nitrophenyl phosphate for the determination of soil phosphodiesterase activity.

The phytase preparation was crude and released orthophosphate from all ester-bonded P compounds. Purified phytase is highly specific to inositol hexakisphosphate (Hayes et al., 2000), so the poor specificity of the phytase preparation used here indicates the presence of other enzymes as impurities. Similar results were obtained by Shand and Smith (1997) and Hayes et al. (2000), who reported the ubiquitous hydrolysis of a range of P compounds by crude preparations of acid phosphomonoesterase and phytase from wheat. Future detailed work on inositol phosphates in soil waters should involve purified

Table 5

Amounts of labile orthophosphate monoesters, orthophosphate diesters and inositol hexakisphosphate present in water extracts of air-dried soils. Values are means of triplicate extracts and are given as $\mu\text{g P g}^{-1}$ soil and as % of the molybdate-unreactive P hydrolysed

Soil no.	Labile orthophosphate monoesters		Orthophosphate diesters		Inositol hexakisphosphate	
	$\mu\text{g g}^{-1}$ soil	% MUP	$\mu\text{g g}^{-1}$ soil	% MUP	$\mu\text{g g}^{-1}$ soil	% MUP
1	< 0.01	< 0.1	0.344	23.0	0.184	12.3
2	< 0.01	< 0.1	0.132	9.0	0.360	24.4
3	0.072	4.8	0.284	17.4	0.348	21.4
4	0.064	4.3	0.216	20.7	0.112	10.8
5	0.020	1.2	0.152	12.3	0.416	33.5

phytase (Hayes et al., 2000). However, for routine studies, the ubiquitous hydrolysis of ester-bound P by the crude phytase preparation allows inositol hexakisphosphate to be estimated by difference from other more specific enzyme preparations.

Phytase activity appeared to be inhibited in extracts of moist soils, which may contribute to the accumulation of inositol hexakisphosphate in soils and its poor availability to plants (Turner et al., 2001). Humic substances are known to cause non-competitive inhibition of phosphomonoesterase (Malcolm and Vaughan, 1979), whilst wheat phytase activity can be reduced to zero in the presence of montmorillonite (Leprince and Quiquampoix, 1996). The lack of inhibition in extracts of dry soils may be due to the disruption of humic compounds through 'tearing' by the physical stresses induced by desiccation (Bartlett and James, 1980).

4.2. Phosphatase hydrolysable phosphorus in soil water-extracts

The small amounts of MUP hydrolysable by alkaline phosphomonoesterase in water-extracts contrasts with previous studies that reported considerably greater proportions of MUP hydrolysis by either acid or alkaline phosphomonoesterase (Fox and Comerford, 1992; Pant et al., 1994; Shand and Smith, 1997). This can be explained by the crude nature of the enzyme preparations used in those studies (similar to the phytase preparation used here), which hydrolyse many organic P compounds including inositol hexakisphosphate and orthophosphate diesters (Shand and Smith, 1997; Hayes et al., 2000). There are considerable inputs of labile orthophosphate monoesters to soils from plants and microorganisms (Webley and Jones, 1971), so their scarcity in soil water-extracts suggests that they must be hydrolysed within a short time of release, as shown by the rapid degradation of labile orthophosphate monoesters added to soils (Bowman and Cole, 1978; Dick and Tabatabai, 1978). This is consistent with the hypothesis that labile organic P turnover is limited by substrate availability rather than by the rate of enzyme activity (Tarafdar and Claassen, 1988), due to the large immobilised phosphomonoesterase component in most soils (Skujins, 1976).

In contrast, large proportions of orthophosphate diesters were detected in water-extracts in the current study, consistent with evidence that they dominate the labile soil organic P pool (Guggenberger et al., 1996). Most organic P inputs to soils are orthophosphate diesters in the form of nucleic acids and phospholipids (Bieleski, 1973; Webley and Jones, 1971) and their prevalence in solution may be due to their slower degradation in soils compared to labile monoesters (Bowman and Cole, 1978; Dick and Tabatabai, 1978).

The amounts of MUP hydrolysed by the enzymes did not exceed 65% in extracts of moist soil and 50% in extracts of dry soil. This is similar to data from previous studies on soil extracts, in which typically <60% of the MUP has been

hydrolysed by (non-specific) phosphatases (Fox and Comerford, 1992; Pant et al., 1994; Shand and Smith, 1997; Hayes et al., 2000; Hens and Merckx, 2001). This non-hydrolysable MUP probably includes live bacteria or cell fragments released by lysis on rewetting of dry soils (Salema et al., 1982), high-molecular weight compounds humic compounds (e.g. Pant et al., 1994; Hens and Merckx, 2001) and P-containing mineral colloids (Kretzschmar et al., 1999).

4.3. Phosphorus release to water by soil drying

The release of large amounts of orthophosphate diesters from dry soils supports the hypothesis that the substantial increases in water-extractable MUP following soil drying are partly due to microbial cell lysis by rapid rewetting (Turner and Haygarth, 2001). However, inositol hexakisphosphate was also released from dry soils in large amounts. This compound is considered to be poorly bioavailable in the soil, because it is protected from enzymatic attack by strong complexation with iron, aluminium and calcium compounds (Turner et al., 2001). Despite this, the release of inositol hexakisphosphate to solution in forms that were amenable to phytase hydrolysis suggests that drying and rapid rewetting may be a mechanism by which this apparently recalcitrant compound becomes available for biological uptake. Solubilisation of inositol hexakisphosphate would presumably occur through disruption of soil aggregates and organic matter by physical stresses induced during the drying and rewetting process. This may have been exacerbated in the current study by soil sieving prior to analysis (Bartlett and James, 1980). However, in situ field soils will also suffer aggregate breakdown during rapid rewetting (Kemper and Rosenau, 1984), especially under conditions likely to generate runoff (i.e. irrigation or heavy rainfall onto dry soils), suggesting that this process will also occur under field conditions.

4.4. Implications for phosphorus transfer from soils to watercourses

Several studies have reported elevated MUP concentrations in drainage water when rainfall followed a dry period (e.g. Livingstone and Whitton, 1984; Turner and Haygarth, 2000), suggesting that the release of organic P when dry soils are rewetted could contribute to P transfer. The functional classification of MUP in soil water-extracts gives insight into the potential impact of MUP mobilisation on surface water quality. For example, labile orthophosphate monoesters would be considered highly bioavailable in watercourses, but their scarcity in soil water-extracts suggests that they constitute only a small proportion of the organic P released to drainage water. This is consistent with the small concentrations of these compounds detected in natural surface waters (typically <5 µg l⁻¹; Shan et al., 1994). In contrast, the prevalence of orthophosphate diesters in water-extracts suggests that they are an important

component of the organic P in soil solution and drainage waters from grassland soils, especially when dry soils are rewet. This has implications for water quality, because orthophosphate diesters are only weakly adsorbed in the soil (Stewart and Tiessen, 1987) and are readily available to blue–green algae in watercourses (Whitton et al., 1991).

Inositol hexakisphosphate is generally considered to have a small P transfer risk due to its strong binding in the soil and poor availability to aquatic microorganisms (McKelvie et al., 1995). However, it was released to water from dry soils in enzyme-hydrolysable forms. It has also been detected in soil leachate water (Espinosa et al., 1999) and suspended river sediments (Suzumura and Kamatani, 1995) and several blue–green algae can use it as a sole P source (Whitton et al., 1991). Therefore, because inositol phosphates represent the major class of organic P in most soils (Turner et al., 2001), their release by soil drying and rapid rewetting may be an important contribution to P transfer and water quality deterioration.

In summary, MUP in soil waters can be conveniently classified into functional groups without the requirement for pre-concentration by measuring the orthophosphate released by commercially-available phosphatase enzymes. Applying this technique to water-extracts of Australian pasture soils revealed the dominance of orthophosphate diesters and the release of (hydrolysable) inositol hexakisphosphate by soil drying and rapid rewetting. Currently, information on the specific P forms in soil solutions and runoff waters is scarce, but is needed to understand the contribution of the MUP fraction in drainage waters to water quality deterioration. The phosphatase technique can provide this information and contribute to improved management strategies for the mitigation of agricultural P pollution.

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